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Investigating the mechanism of glyphosate resistance in rigid ryegrass (*Lolium ridigum*)

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Glyphosate is a broad-spectrum herbicide that has been used extensively for more than 20 yr. The first glyphosate-resistant weed biotype appeared in 1996; it involved a rigid ryegrass population from Australia that exhibited an LD_{50} value approximately 10-fold higher than that of sensitive biotypes. We have characterized gene expression levels and glyphosate sensitivity of 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS), the target enzyme for glyphosate inhibition, in sensitive and resistant lines derived from this population. Restriction fragment length polymorphism analyses were also performed to examine the distribution of EPSPS gene variants and the gene copy number. A two- to threefold increase in basal EPSPS messenger RNA (mRNA) and enzyme activity levels was observed in the most resistant lines analyzed; however, differences among lines in the sensitivity of EPSPS to glyphosate were not apparent. Induction of EPSPS was observed within 48 h after application of 1.5 kg ae ha^{-1} of glyphosate. This was reflected in elevated levels of both EPSPS mRNA and enzyme activity. Similarly, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase mRNA levels increased after glyphosate treatment; however, basal and induced transcript levels were comparable for sensitive and resistant lines in this case. The restriction fragment length polymorphism analyses showed no evidence for gene amplification or cosegregation of a specific EPSPS gene variant with glyphosate resistance. EPSPS expression in lines exhibiting an intermediate level of resistance was indistinguishable from that in glyphosate-sensitive lines, suggesting that the mechanism could, at least in part, be non–target-based.

Nomenclature: Glyphosate; rigid ryegrass, *Lolium rigidum* Gaudin LOLRI.

Key words: DAHPS, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase, 5-enolpyruvylshikimate 3-phosphate synthase, EPSPS, herbicide resistance.

Rigid ryegrass is an annual, diploid grass native to the Mediterranean and has become one of the most widely established grass weeds within the cropping belt of southern Australia (Powles et al. 1998). Although rigid ryegrass is extensively cultivated as a pasture plant, its presence within crops can lead to dramatic yield reductions if it is not controlled (Holtum and Powles 1991). Rigid ryegrass has evolved resistance to numerous classes of herbicides with distinct modes of action (Hall et al. 1994; Preston et al. 1996) and can also exhibit cross-resistance to multiple classes (e.g., Powles et al. 1990). The ability of rigid ryegrass to accumulate resistance mechanisms and acquire cross-resistance has been attributed to its widespread distribution within cropping regions, prolific seed set, allogamous reproductive habit, as well as significant genetic variability and phenotypic plasticity (Powles and Matthews 1992).

Evaluation of resistance risk criteria and current and historical use data have led to the conclusion that glyphosate is at low risk for the development of weed resistance (Bradshaw et al. 1997). This notion has been supported in theory by saturation mutagenesis experiments conducted with Arabidopsis (*Arabidopsis thaliana* Heynh.), where efforts to isolate glyphosate-resistant mutants from ethylmethane sulfonate–mutagenized populations have been unsuccessful, whereas mutants exhibiting high levels of tolerance to two different acetolactate synthase–inhibiting herbicides were readily obtained using identical screening procedures (Haughn and Somerville 1986, 1987, 1990). Nevertheless, two recent reports have documented the appearance of glyphosate-resistant rigid ryegrass in New South Wales, Australia. These instances occurred within an isolated field (Pratley et al. 1996, 1999) and within an orchard (Powles et al. 1998) subjected to repeated applications of glyphosate for 15 yr and represent the first examples of apparent evolved resistance to glyphosate in any weed species. Subsequent to these reports, glyphosate-resistant goosegrass (*Eleusine indica* L. Gaertn.) biotypes have also been discovered in Malaysia (Lee and Ngim 2000; Tran et al. 1999).

Previous studies involving the glyphosate-resistant rigid ryegrass biotype collected in Echuca, New South Wales, Australia, showed that the resistant biotype exhibits up to 10-fold increase in resistance compared with the susceptible biotype (Pratley et al. 1999) and concluded that altered uptake, translocation, or metabolism of glyphosate was not associated with the mechanism of resistance (Feng et al. 1999). In this article, we extend this work by comparing basal and glyphosate-induced 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) enzymatic activity and steady-state messenger RNA (mRNA) levels in resistant and sensitive lines as well as by examining the glyphosate sensitivity of the enzyme(s) expressed in different lines. We also present restriction fragment length polymorphism data to determine whether resistance cosegregates with a specific EPSPS variant and analyses of in vivo shikimate levels to determine whether the herbicide is excluded from its target site. We report evidence, at both the enzymatic activity and mRNA levels,

of increased EPSPS expression for the most highly resistant lines within the population analyzed and discuss the possible significance of these observations relative to the levels of whole-plant glyphosate resistance exhibited.

Materials and Methods

EPSPS Enzyme Activity Levels in Rigid Ryegrass Lines

Seed were from bulked progeny of a population of rigid ryegrass (designation 48118a, Pratley et al. 1999), which included survivors of a glyphosate application equivalent to 1.73 kg ae ha^{-1} in greenhouse experiments. Plants were maintained in a greenhouse with approximate day and night temperatures of 27 and 20 C, respectively, 60 to 90% relative humidity, and natural lighting supplemented with artificial lighting to provide a total day length of 14 h and a minimum of 500 μmol m^{-2} s^{-1}. Clones of individual plants were generated by removing 10 to 20 tillers and subtending root mass per plant, which were then repotted.

Herbicide applications were made using an automated cabinet sprayer equipped with a moving boom. Solutions were applied using a 8001E TeeJet tip[1] at a boom height of 40 cm delivering a diluent volume of 225 L ha^{-1} at a pressure of 186 kPa. Plant evaluations were conducted 3 wk posttreatment.

To screen 48118a progeny for glyphosate resistance, 125 seeds (hereafter referred to as "lines") were grown for 8 wk, and four clones were generated from each line and treated with 0.0, 0.5, 1.5, or 3.0 kg ae ha^{-1} formulated (isopropylamine salt) glyphosate 1 wk after repotting. Some clones showed rapid development of chlorosis and necrosis (scored visually) and did not survive the treatments. Others exhibited significant stem and leaf necrosis and transitory tiller chlorosis; however, new growth was free of symptoms. The latter were considered "survivors" of a given treatment, the former "nonsurvivors." On the basis of these preliminary screens, lines were assigned to four different resistance "classes": those that survived 0.5, 1.5, or 3.0 kg ae ha^{-1} treatments were designated as intermediate resistant (I), resistant (R), or highly resistant (H), respectively. Lines that did not survive 0.5 kg ae ha^{-1} treatments were designated as glyphosate-sensitive (S). The untreated plants from 32 selected lines were further subdivided into four new clones and were grown for an additional 4 wk before use in molecular and biochemical studies. For these studies, two clones per line were treated with 1.5 kg ae ha^{-1} glyphosate plus 0.15% MON 0818 surfactant,[2] and two were administered a control treatment consisting of surfactant alone. Crown tissues were then harvested at $t = 0$ h and $t = 48$ h after treatment from both treated and control experimental groups, and leaf tissues were collected for genomic DNA isolations (described below).

Extracts were prepared from 2 g of crown tissue pulverized using a mortar and pestle in liquid nitrogen. All manipulations were performed at 4 C unless otherwise noted. The powdered tissues were placed in 250-ml disposable plastic bottles containing 100 ml of extraction buffer (100 mM Tris HCl, pH 7.5, 10% glycerol [v/v], 1 mg ml^{-1} bovine serum albumin [BSA], 1 mM ethylenediaminetetraacetic acid [EDTA], 1 mM benzamidine, and 1 mM dithiothreitol). In addition, 500 mg of polyvinylpolypyrrolidone was added to each sample before extraction. Each sample was homogenized for 2 min at 8,000 rpm using a PT3000 Polytron homogenizer,[3] and extracts were filtered through a Millipore 0.45-μm filter[4] lined with a prefilter and Miracloth®[5] to prevent clogging. Extracts were precipitated overnight by gradual addition of solid ammonium sulfate to a concentration of 80% (w/v) with gentle stirring, then centrifuged in 50-ml Oakridge tubes for 30 min at 10,000 × *g*. Pellets were resuspended in 15 ml of extraction buffer, and approximately 2.5 ml was passed through a PD-10 desalting column.[6] The solution was then refiltered through a 0.2-μm prefilter cartridge[7] and was concentrated approximately 10-fold using an Ultrafree #4 centrifugal filter[8] as per the manufacturer's instructions. Glycerol was then added to the samples to 10% (v/v). Protein concentrations were determined using the method of Bradford (1976) with BSA as the standard. Extracts were stored at −20 C before the assay.

EPSPS specific activity levels were determined using a radiometric high-performance liquid chromatograpy (HPLC)–based assay as described by Padgette et al. (1988). Ten-microliter samples of extract were assayed in a total volume of 50 μl of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.0), 0.1 mM ammonium molybdate, 5 mM potassium fluoride, 0.5 mM ^{14}C-labeled phosphoenolpyruvate (^{14}C-PEP)[9] (specific activity, 1.073 GBq $mmol^{-1}$), and 1 mM shikimate-3-phosphate for 2 to 15 min at 25 C, then quenched by the addition of 50 μl of 100% ethanol–0.1 N acetic acid (9:1 v/v), pH 4.9, before HPLC analysis. Shikimate-3-phosphate was prepared as described by Marzabadi et al. (1996). For ^{14}C-lableled 5-enolpyruvylshikimate-3-phosphate (^{14}C-EPSP) detection, 30 μl of the quenched reactions was injected into a Synchropak AX100 anion exchange column[10] equilibrated with 0.235 M K_3PO_4 buffer (pH 6.5) and was eluted isocratically with the same buffer.

EPSPS and 3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase Steady-State mRNA Levels

Total RNA was isolated from 50 mg flash-frozen, pulverized crown tissues using an RNeasy® Plant Mini-Kit,[11] modified by an additional homogenization step of 30 s at 25,000 rpm using a handheld homogenizer.[12] RNA recovery and purity were determined spectrophotometrically, and sample integrity was assessed by agarose gel electrophoresis. Northern blotting procedures were performed according to standard protocols (Sambrook et al. 1989) using a ^{32}P-labeled 1.0-kb partial rigid ryegrass EPSPS complementary DNA (cDNA) (GenBank accession number, AF349754) or 0.8-kb partial corn (*Zea mays* L.) 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS) cDNA (GenBank accession number BG370023) as the hybridization probe. cDNA probes were radiolabeled to a specific activity of at least 120 GBq μg^{-1} using the Rediprime II® random-prime labeling system[13] according to manufacturer's instructions. Equivalent sample loadings were confirmed by ethidium bromide staining of the gels before membrane transfer. Membranes were washed twice at room temperature in 0.25% (w/v) sodium dodecyl sulfate (SDS) and 2× standard saline citrate (SSC; 1× SSC contains 0.15 M NaCl and 15 mM sodium citrate), followed by one or two washes at 55

C (EPSPS probe) or 45 C (DAHPS probe) in 0.25% (w/v) SDS and 0.2× SSC. Hybridization signal intensities were quantified using a Storm® phosphoimager.[14] For reprobing of Northern blots, membranes were stripped in 0.1% (w/v) SDS at 80 C for 20 to 30 min and examined for complete probe removal by autoradiography.

Inhibition of EPSPS Activity by Glyphosate

EPSPS assays were performed in the manner described above, except that reactions were performed both in the presence and absence of 1.6 μM (isopropylamine salt) glyphosate. Percent inhibition by glyphosate was calculated by taking the ratios of ^{14}C-PEP incorporation in assays performed using identical extracts.

Comparison of EPSPS Gene Families in (S) and (H) Lines

Genomic DNA was isolated from 300 to 400 mg lyophilized rigid ryegrass leaves using the method of Saghai-Maroof et al. (1984) and resuspended in 300 μl TE (10 mM Tris–HCl, pH 7.5, 1 mM EDTA). DNA recovery and purity were determined spectrophotometrically, and sample integrity was assessed by agarose gel electrophoresis.

Ten micrograms of DNA was digested with *Ssp*I, *Sph*I, *Spe*I, *Eco*RV, *Bsp*HI, or *Dra*I, and Southern blotting procedures were performed according to standard protocols (Sambrook et al. 1989) using a ^{32}P-labeled rigid ryegrass EPSPS cDNA (described above). Equivalent sample loadings and complete restriction endonuclease digestions were confirmed by ethidium bromide staining of the gels before membrane transfer. Membranes were washed as described above for Northern analysis, except that SDS was used at 0.2% (w/v).

Shikimate Accumulation in Response to Glyphosate Treatment

Shikimate levels in crown tissue samples were determined using a modification of the procedures of Höllander-Czytko and Amrhein (1983) and Singh and Shaner (1998). Approximately 100 to 200 mg of lyophilized, ground tissue was homogenized in 1 ml of 0.25 N sulfuric acid using a handheld homogenizer and filtered through a 0.2-μm Acrodisc filter.[15] Fifty microliters of the filtrate was analyzed by a 717 HPLC system[16] using a C18 reversed-phase column.[17] The mobile phase was 1% methanol in 20 mM phosphate buffer (pH 2.2) for 5 min, followed by 10% methanol for 5 min; shikimate eluted as a single peak at approximately 5.2 min using a flow rate of 1 ml min^{-1}. The eluate was monitored at 210 nm using a photodiode array detector.[18] Peak areas and standard curve calculations were determined using the Millenium v2.15 HPLC software package.[19] Standard curves were generated using known quantities of shikimate in 0.25 N sulfuric acid. The limit for detection of shikimate in crown tissues was approximately 1.0 micrograms per gram of tissue (fresh weight) using this method.

Results and Discussion

EPSPS Enzyme Activity Levels in Rigid Ryegrass Lines

Rigid ryegrass has been categorized as an obligate outcrossing species (Naylor 1960), and thus a high degree of genetic diversity would be expected among lines within the rigid ryegrass 48118a population. Because of this presumed heterogeneity, multiple (S), (I), (R), and (H) lines were analyzed to determine whether high levels of glyphosate resistance were associated with increased EPSPS activity levels (Figure 1).

Increases in EPSPS activity levels were observed among the (S), (R), and (H) lines 48 h after glyphosate treatment (Figure 1). The degree of induction of EPSPS activity observed was similar overall, the activity increasing between two- and threefold relative to basal (0 h after treatment) levels. Induction of EPSPS was not clearly discernible for (I) lines from these data. Comparison of EPSPS activity among the four resistance classes at 48 h posttreatment indicates approximately 2.5- to 3.5-fold higher activity levels in the (R) and (H) lines relative to the (I) and (S) lines. Mock (surfactant-only) treatments clearly showed that the observed induction of EPSPS was because of the presence of glyphosate.

EPSPS and DAHPS Steady-State mRNA Levels

The comparison of glyphosate-induced EPSPS mRNA levels among different resistance classes was in general agreement with the comparison of enzyme activity levels shown in Figure 1; however, basal (0 h post–glyphosate application) EPSPS mRNA levels were also higher in the (R) and (H) lines than in the (I) and (S) lines (Figure 2). Basal transcript levels observed in (R) and (H) lines were approximately 2.5- to threefold higher than those observed in (I) and (S) lines. Increases in transcript levels were observed for all resistance classes 48 h after glyphosate application. In (R) and (H) lines, the induction was slightly less than twofold, and in (S) and (I) lines, the induction was slightly greater than twofold (Figure 2B). As was observed for enzyme activity levels (Figure 1), lines exhibiting intermediate resistance levels were indistinguishable from glyphosate-sensitive lines at both 0 and 48 h. Significant changes in steady-state mRNA levels were absent in mock (surfactant-only) treatments. The magnitude of increases observed after glyphosate treatment was similar for the levels of steady-state mRNA accumulation and enzyme activity. Therefore, the simplest interpretation would be that increased EPSPS activity results directly from increased mRNA levels.

Blots used for analysis of EPSPS transcript levels were reprobed to monitor levels of DAHPS mRNA, which corresponds to the first committed enzymatic step in the shikimate pathway (reviewed by Herrmann 1995). In contrast to EPSPS, elevated DAHPS transcript levels did not appear to be associated with the (R) and (H) lines as compared with the (I) and (S) lines at either 0 or 48 h (Figure 3). As was seen for EPSPS, increases in DAHPS transcript levels were observed for all groups in response to glyphosate treatment by 48 h, ranging from an approximately 1.5- to 2.5-fold induction, relative to basal (0 h) levels (Figure 3B). The

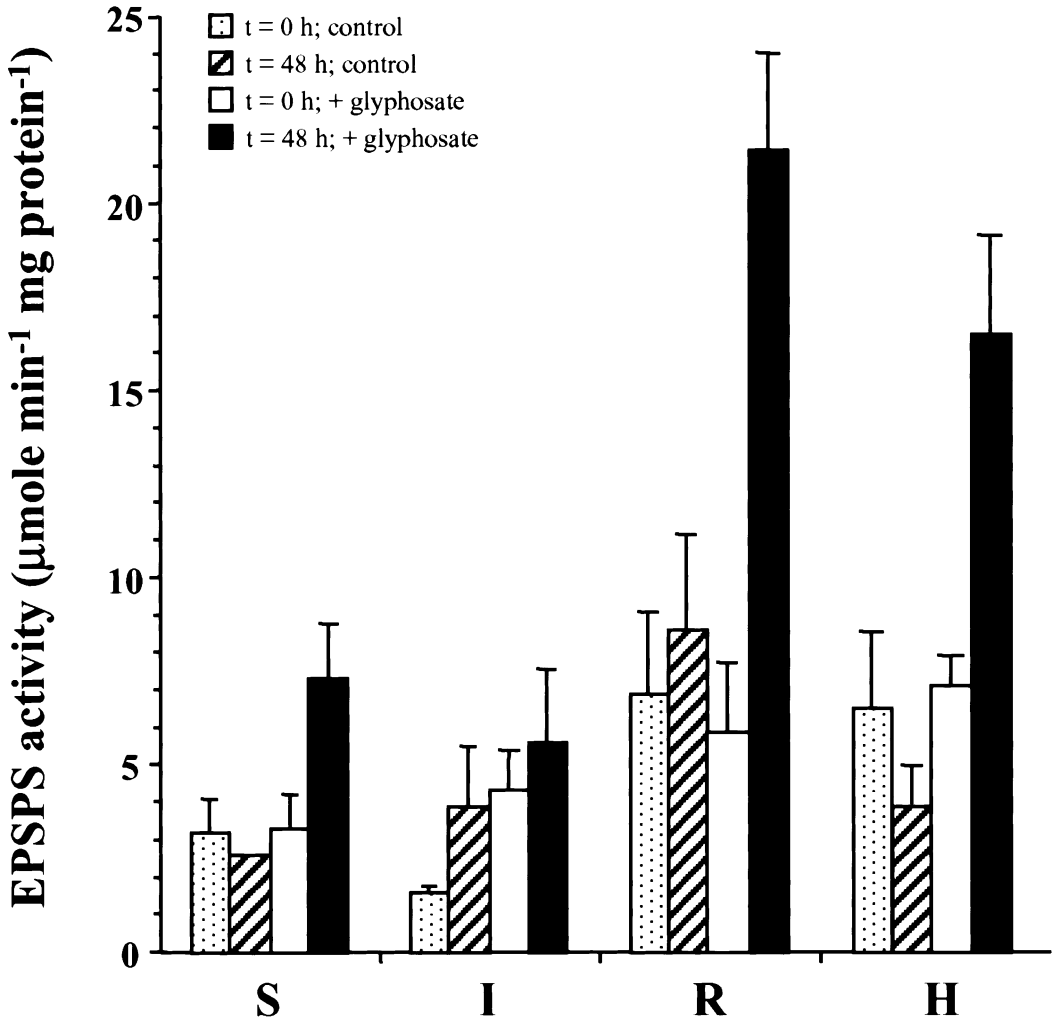

FIGURE 1. Basal and glyphosate-induced 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) activities in glyphosate-sensitive (S), intermediate resistant (I), resistant (R), and highly resistant (H) lines. Extracts prepared from crown tissues were assayed for EPSPS activity. Two clones per line were spray-treated with glyphosate (+ glyphosate), and the remaining two received surfactant-only control applications (control). All assays were performed in triplicate. $n = 2$ for the (S) "$t = 48$ h, control" treatment; otherwise each bar represents the mean activity observed within at least three lines per resistance class. Vertical lines indicate the standard error.

increase of DAHPS mRNA levels in response to glyphosate is consistent with earlier studies using potato (*Solanum tuberosum* L.) cell suspension cultures (Herrmann 1995; Pinto et al. 1988) where a severalfold induction of both DAHPS mRNA and protein was detected within 24 h of exposure to the herbicide.

The finding that increased EPSPS expression appears to cosegregate with high levels of resistance among the rigid ryegrass 48118a progeny suggests that EPSPS overexpression plays some role in the resistance mechanism. Numerous examples of glyphosate resistance occurring from whole plant selection or selection from cell suspensions have been attributed to overexpression of EPSPS (reviewed by Bradshaw et al. 1997). In resistant carrot (*Daucus carota* L.), tobacco (*Nicotiana tabacum* L.), and petunia (*Petunia hybrida* L.) cell lines, molecular characterization has revealed that overexpression is caused by specific amplification of EPSPS genes (Jones et al. 1996; Shah et al. 1986; Suh et al. 1993). In studies performed by Boerboom et al. (1990) involving nine different birds foot trefoil (*Lotus corniculatus* L.) selections, a threefold range in glyphosate tolerance among the accessions was correlated with a 2.6-fold increase in EPSPS specific activity, although molecular characterization of the accessions was not done.

The observation that increased levels of both EPSPS enzyme activity and mRNA occur within 48 h of exposure to glyphosate is also of interest because these data may support the notion that the biosynthetic step catalyzed by EPSPS serves as a transcriptional control point within the shikimate pathway. Previous work using tomato (*Lycopersicon esculentum* L.) cell suspension cultures has demonstrated rapid increases in EPSPS transcript levels in response to fungal elicitors (Görlach et al. 1995); however, the effects of specific shikimate pathway inhibitors, such as glyphosate, on EPSPS transcript levels have not been reported previously. It should be noted that the present study does not necessarily provide incontrovertible evidence for EPSPS being subject to transcriptional feedback control because wounding may also induce shikimate pathway mRNAs (e.g., Dyer et al. 1989), and the present experiments were not designed to differentiate between herbicide-induced injury and reduced shikimate pathway flux.

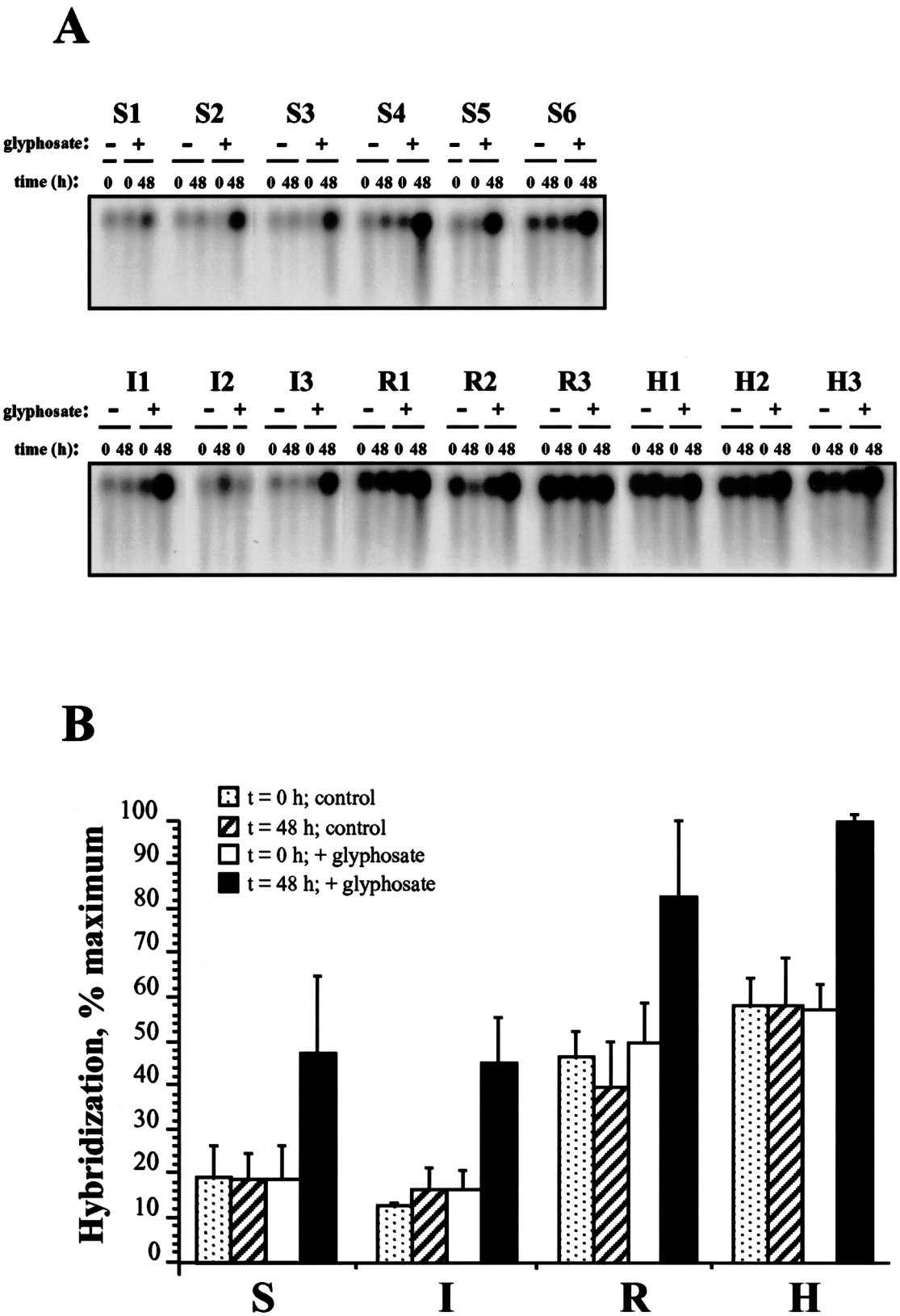

FIGURE 2. Northern analysis of basal and induced 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) levels in glyphosate-sensitive (S), intermediate resistant (I), resistant (R), and highly resistant (H) lines. (A) Blots hybridized using a radiolabeled rigid ryegrass (*Lolium rigidum*) EPSPS complementary DNA probe. (B) Hybridization signals from (A) quantitated using a phosphoimager. Values were normalized as "percent maximum" with (H) "t = 48 h, + glyphosate" set to 100%. n = 2 for the (I) "t = 48 h, + glyphosate" treatment; otherwise each bar represents the mean signal intensity generated from at least three lines per resistance class, as shown in (A). Vertical lines indicate the standard error.

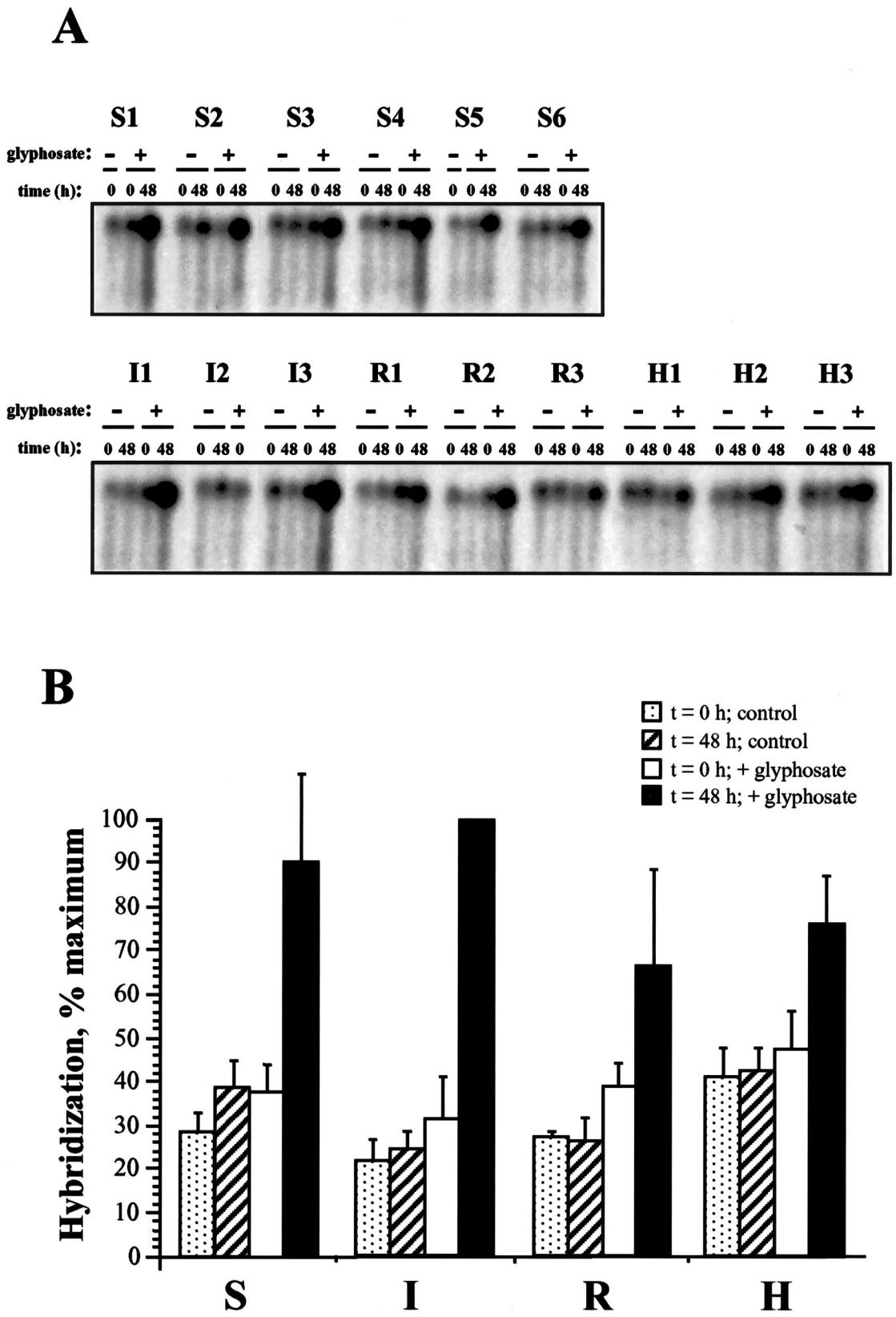

FIGURE 3. Northern analysis of basal and induced 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS) levels in glyphosate-sensitive (S), intermediate resistant (I), resistant (R), and highly resistant (H) lines. (A) Blots hybridized using a radiolabeled corn (*Zea mays* L.) DAHPS complementary DNA probe. (B) Hybridization signals from (A) quantitated as described in Figure 2. Vertical lines indicate the standard error.

TABLE 1. Glyphosate sensitivity of basal and induced 5-enolpyruvylshikimate 3-phosphate synthase activity pools in (S), (I), (R), and (H) lines.[a] Percent inhibition of ^{14}C-labeled phosphoenolphyruvate turnover by 1.6 μM glyphosate was determined for crown tissue extracts prepared from lines at $t = 0$ and 48 h post-application of 1.5 kg ae ha^{-1} glyphosate.

	% Inhibition by 1.6 μM glyphosate[b]	
Class	$t = 0$ h	$t = 48$ h
(S)	42.9 ± 4.0	38.3 ± 7.4
(I)	44.3 ± 3.0	42.4 ± 8.8
(R)	42.2[c]	43.6 ± 2.9
(H)	36.6 ± 6.5	44.5 ± 2.8

[a] Lines were assigned to four different resistance "classes": those that survived up to 0.5, 1.5, or 3.0 kg ae ha^{-1} glyphosate treatments were designated as intermediate resistant (I), resistant (R), or highly resistant (H), respectively. Lines that did not survive 0.5 kg ae ha^{-1} treatments were designated as glyphosate-sensitive (S).

[b] Values are mean ± SE; $n = 3$ to 6 lines, unless indicated.

[c] $n = 2$.

Inhibition of EPSPS Activity by Glyphosate

EPSPS specific activities were determined in the presence and absence of 1.6 μM glyphosate for (S), (I), (R), and (H) lines (Table 1), and the percent inhibition by glyphosate was calculated from these values. This analysis was performed at 0 and 48 h (post–glyphosate application) to compare sensitivities of both the basal and glyphosate-induced EPSPS activity pools. The results of these experiments suggest that glyphosate resistance within the 48118a population is not associated with reduced sensitivity of the respective EPSPS enzyme pools to glyphosate (Table 1). Percent inhibition of ^{14}C-PEP conversion to ^{14}C-EPSP by glyphosate was similar in all the lines analyzed, ranging from approximately 37 to 44%. In almost all cases, the observed differences fell within the range of experimental error. The consistency of the data from sample to sample indicates that sufficient amounts of inhibitor were present such that the values obtained were not influenced by modest differences in EPSPS specific activity levels (Figure 1). In fact, the glyphosate concentration used was chosen to be at approximately the $I_{50,glyphosate}$, where the greatest sensitivity to inhibition occurs. Thus, a change in inhibition kinetics caused by an EPSPS variant, if present, would likely have been detected. Even so, we cannot eliminate the possibility that a resistant EPSPS activity constituted a minor percentage of the overall activity pool in some or all of the resistant lines analyzed because detailed dose–response curves were not generated for each extract. These results, however, clearly indicate that resis-

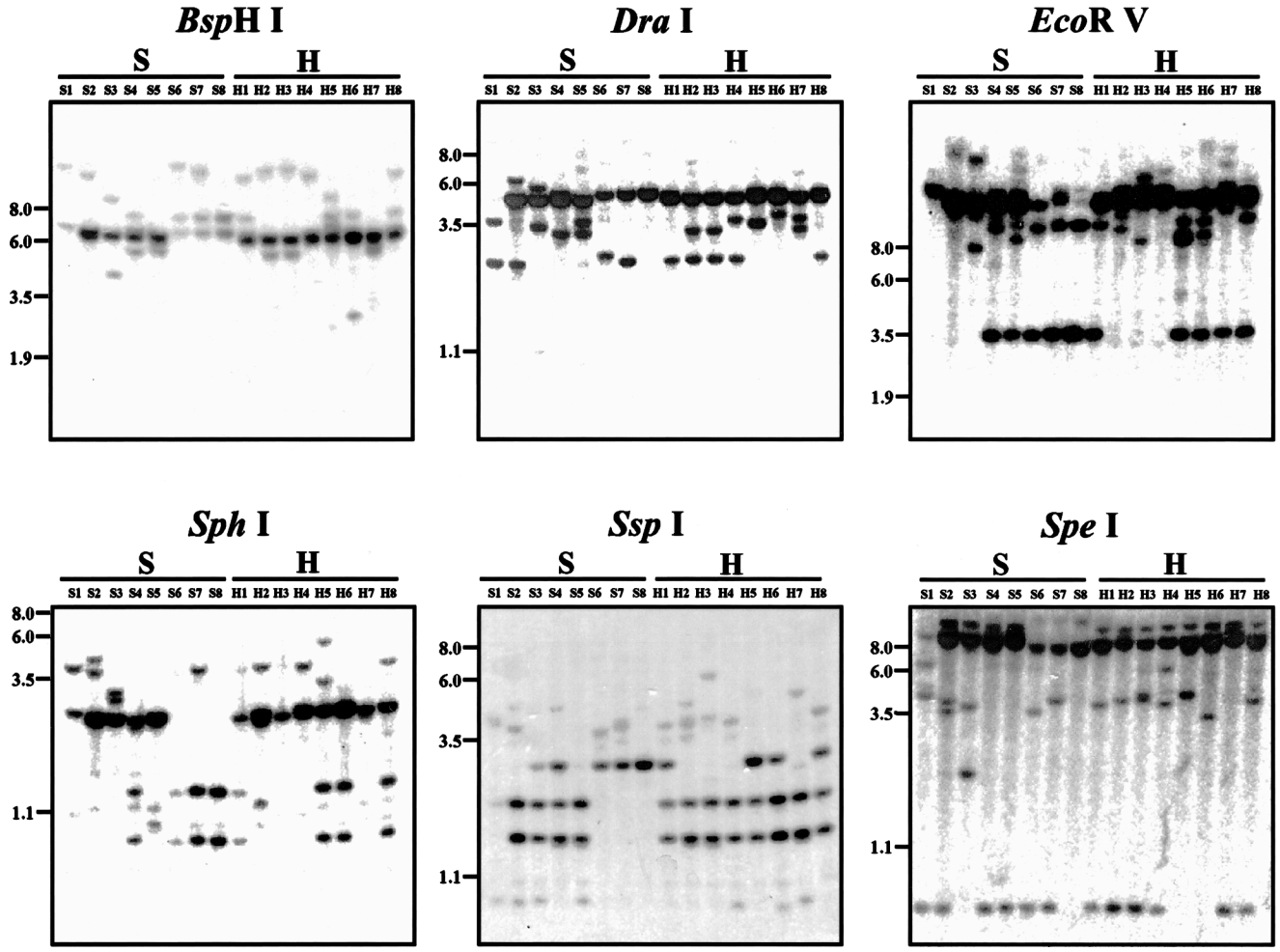

FIGURE 4. Southern analysis of glyphosate-sensitive and -resistant rigid ryegrass (*Lolium rigidum*) lines. Genomic DNA prepared from eight different glyphosate-sensitive (S) and highly resistant (H) lines was digested with *Bsp*HI, *Dra*I, *Eco*RV, *Sph*I, *Ssp*I, or *Spe*I, then hybridized with a radiolabeled rigid ryegrass 5-enolpyruvylshikimate 3-phosphate synthase complementary DNA probe.

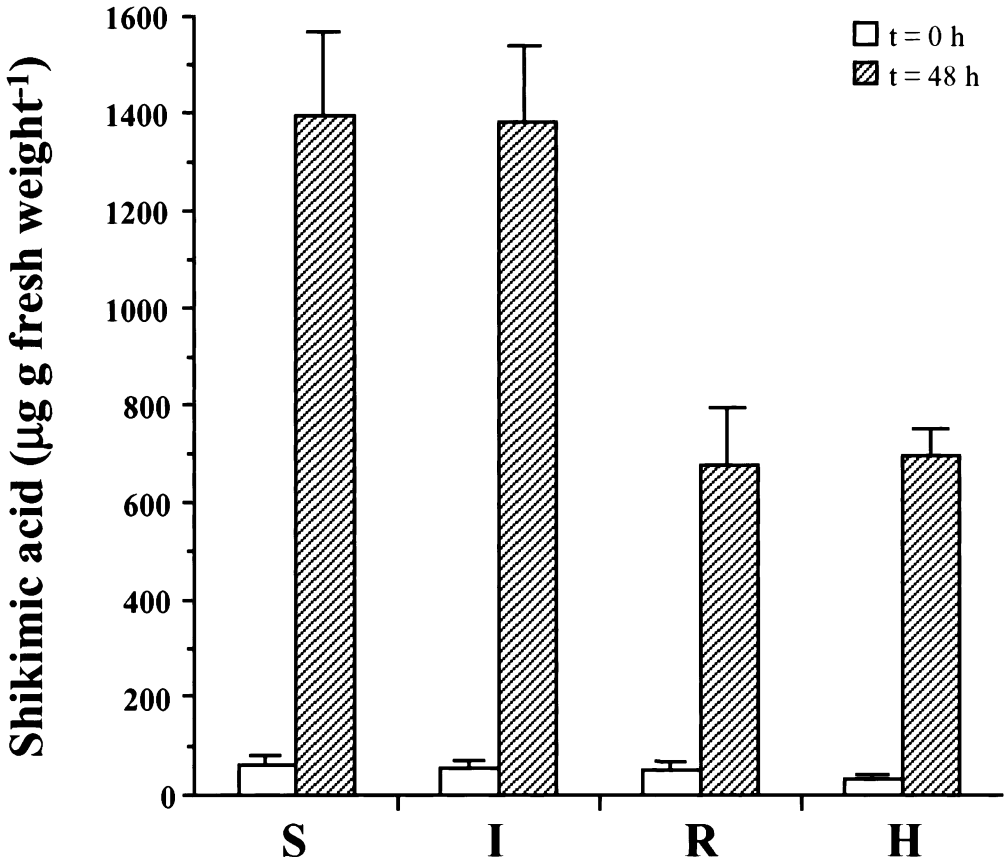

FIGURE 5. Shikimic acid levels in glyphosate-sensitive (S), intermediate resistant (I), resistant (R), and highly resistant (H) lines after exposure to glyphosate. Shikimic acid levels were determined by high-performance liquid chromatography at 0 and 48 h post–glyphosate treatment. All analyses were performed from triplicate samples; each bar represents mean levels observed within at least three lines per resistance class. Vertical lines indicate the standard error.

tance is not associated with a predominant, glyphosate-insensitive EPSPS activity.

Previous in vitro mutagenesis studies demonstrated the ability to generate plant-derived EPSPS variants that confer glyphosate resistance to transformed plants (reviewed by Bradshaw et al. 1997). The corresponding mutations, however, tend to impart a significant reduction in catalytic efficiency of the enzyme (Padgette et al. 1991). This has in fact been proposed as one underlying explanation for the infrequency with which glyphosate resistance has evolved in weed species (Bradshaw et al. 1997). Nevertheless, the recent discovery of a glyphosate-resistant EPSPS enzyme from goosegrass (Baerson et al. 2002) raises the possibility that glyphosate-resistant weed biotypes can evolve by means of alterations in the coding regions of EPSPS genes.

Comparison of EPSPS Gene Families in (S) and (H) Lines

Changes in the EPSPS expression levels in glyphosate-resistant rigid ryegrass could potentially be caused by gene structural alterations or amplification of one or more EPSPS gene family members. To examine this possibility, EPSPS-related DNA polymorphisms were examined for eight (H) and eight (S) lines using six different restriction endonucleases (Figure 4).

Among the lines analyzed, the data indicate that EPSPS comprises a small multigene family of about two to five members. Polymorphisms were detected from all the digests performed; however, none of these appeared to be associated specifically with the (H) lines. In the case of gene amplification, an increase in the intensity of specific bands or the presence of a novel band specific to the (H) lines would be expected. But this was not observed for any of the digests performed. Given the number of different enzymes used, coupled with the fact that a nearly full-length EPSPS cDNA clone was used to generate hybridization probes, it is likely that novel polymorphisms would have been detected had gene amplification occurred.

As previously mentioned, amplified EPSPS loci have been identified in glyphosate-resistant cell lines and regenerated plants (Jones et al. 1996), although the conditions considered permissive for gene amplification, such as maintenance of chromosomal deletions and broken chromosomes, seem to be specific to cultured plant cells (reviewed by Peschke and Phillips 1992; Smith et al. 1995). In contrast, these conditions do not normally exist in whole organisms, with the exception of some specialized cell types. Hence, the possibility for glyphosate resistance occurring spontaneously by this mechanism in weed species would seem somewhat remote.

In the event that an EPSPS allele had arisen with an altered promoter or other regulatory region, the variant would be present in all (H) lines and may be identifiable as an EPSPS-related polymorphism. As mentioned, this was not observed in our study; however, it should be noted that point mutations and small insertions and deletions would not be detected by Southern analyses if specific endonuclease

target sites were unaltered. Overall, the EPSPS-related polymorphisms observed were common to both (S) and (H) lines, suggesting a random distribution of EPSPS variants as a result of outcrossing among plants of similar origin.

Shikimate Accumulation in Response to Glyphosate Treatment

The ability of EPSPS to maintain a flux through the shikimate pathway in vivo after glyphosate exposure was investigated in (S), (I), (R), and (H) lines by analyzing shikimate levels 0 and 48 h posttreatment (Figure 5). Because it has been previously shown that inhibition of EPSPS by glyphosate results in the accumulation of shikimate rather than shikimate-3-phosphate (Höllander-Czytko and Amrhein 1983; Singh and Shaner 1998), the latter compound was not quantified.

Similar basal levels of shikimate were seen within (S), (I), (R), and (H) lines, ranging from approximately 35 to 60 $\mu g\ g^{-1}$ fresh weight. Although shikimate levels increased more than 10-fold in all lines 48 h after glyphosate application, significant differences were observed. In (S) and (I) lines, similar levels of shikimate accumulated after glyphosate treatment, averaging approximately 1,400 $\mu g\ g^{-1}$ fresh weight, whereas approximately one-half of this amount accumulated in (R) and (H) lines. Interestingly, plants representing class (I) were indistinguishable from glyphosate-sensitive (S) lines, as was the case observed for EPSPS enzymatic activity (Figure 1) and steady-state mRNA levels (Figure 2).

The results shown in Figure 5 demonstrate an approximately twofold enhanced capacity of the (R) and (H) lines to maintain carbon flux through the shikimate pathway in the presence of glyphosate. Furthermore, the greater than 10-fold increase in shikimate levels in (R) and (H) lines indicates that the herbicide is not excluded from its target site in vivo and that it has a significant effect on carbon flow through the pathway. It is interesting to note that in experiments performed by Singh and Shaner (1998) on glyphosate-treated soybean [*Glycine max* (L.) Merr.], no accumulation of shikimate was evident in soybean transformed with a glyphosate-insensitive EPSPS, whereas a large accumulation of shikimate was observed in glyphosate-treated, nontransgenic soybean. It is therefore unlikely that the efficacy of the resistance mechanism operating in rigid ryegrass is similar to that of the mechanism currently being used in commercially developed glyphosate-resistant crops, although in the latter case, glyphosate-insensitive EPSPS genes are highly overexpressed (e.g., Shah et al. 1988).

Whereas the correlation between high levels of glyphosate tolerance and elevated EPSPS mRNA and activity levels in (H) and (R) lines is intriguing, the extent to which two- to threefold differences could contribute to the observed 10-fold increase in resistance (Pratley et al. 1999) is not clear. Cosegregation of EPSPS overexpression with resistance in rigid ryegrass, coupled with the demonstration of monogenic inheritance will be necessary to conclude that the two- to threefold EPSPS overexpression is the sole determinant involved. In addition, the possibility for segregants within the population exhibiting intermediate (I) levels of tolerance in the absence of EPSPS overexpression (Figures 1 and 2) would argue in favor of a more complex mechanism. Similar conclusions were drawn in the case of glyphosate-resistant field bindweed (*Convolvulus arvensis* L.) (Duncan and Weller 1987; Westwood and Weller 1997), where several distinct processes including elevated shikimate pathway activity, differential induction of EPSPS activity by glyphosate, as well as reduced herbicide uptake may be involved in the resistance mechanism.

Sources of Materials

[1] 8001E TeeJet tip, Spraying Systems Co., P.O. Box 7900, Wheaton, IL 60189.

[2] MON 0818 surfactant, Monsanto Co., 700 Chesterfield Parkway North, St. Louis, MO 63198.

[3] PT3000 Polytron homogenizer, Brinkman Instruments Inc., One Cantiague Rd., P.O. Box 1019, Westbury, NY 11590.

[4] Millipore 0.45-μm filter, Millipore Corporation, 80 Ashby Road, Bedford, MA 01730.

[5] Miracloth®, Calbiochem, P.O. Box 12087, La Jolla, CA 92039.

[6] PD-10 desalting column, Amersham Pharmacia Biotech Inc., 800 Centennial Avenue, P.O. Box 1327, Piscataway, NJ 08855.

[7] 0.2-μm prefilter cartridge, Millipore Corporation, 80 Ashby Road, Bedford, MA 01730.

[8] Ultrafree #4 centrifugal filter, Millipore Corporation, 80 Ashby Road, Bedford, MA 01730.

[9] ^{14}C-PEP, Amersham Pharmacia Biotech Inc., 800 Centennial Avenue, P.O. Box 1327, Piscataway, NJ 08855.

[10] Synchropak AX100 anion exchange column, cat.# 942804, P.J. Cobert Associates, Inc., P.O. Box 460046, St. Louis, MO 63146.

[11] RNeasy® Plant Mini-Kit, Qiagen Inc., 28159 Avenue Stanford, Valencia, CA 91355.

[12] Handheld homogenizer, model PT1200CL, Brinkman Instruments Inc., One Cantiague Rd., P.O. Box 1019, Westbury, NY 11590.

[13] Rediprime II® random-prime labeling system, Amersham Pharmacia Biotech Inc., 800 Centennial Avenue, P.O. Box 1327, Piscataway, NJ 08855.

[14] Storm® phosphoimager, Molecular Dynamics, 928 East Arques, Sunnyvale, CA 94086.

[15] 0.2-μm Acrodisc filter, Pall Life Sciences, 600 South Wagner Road, Ann Arbor, MI 48103.

[16] 717 HPLC system, Waters Corp., 34 Maple Street, Milford, MA 01757.

[17] C18 reversed-phase column, 250 × 4.6 mm, Alltech Associates Inc., 2051 Waukegan Road, Deerfield, IL 60015.

[18] Photodiode array detector, model #966, Waters Corp., 34 Maple Street, Milford, MA 01757.

[19] Millenium v2.15 HPLC software package, Waters Corp., 34 Maple Street, Milford, MA 01757.

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